# THE WILLIAM ALLAN MEMORIAL AWARD LECTURE

## Tadpoles, Chicks, and Red Cells: A Study of Development

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Dr. Glass, Dr. Motulsky, ladies and gentlemen: I feel very honored that you have given me the William Allan Memorial Award, and I would like to thank all of you and the Committee of the Society. I have been quite scared to approach or to attempt to join the American Society of Human Genetics, since I do not qualify under any part of its name except perhaps that I am human!

As Arno Motulsky has pointed out, many people were involved with this work, and its success is in large measure due to them. I would like to mention particularly Max Perutz, Francis Crick, Herman Lehmann, and also Jim Neel, who was honored by your Society in 1965. I have been very fortunate in my collaborators: C. Baglioni, L. Bernini, B. Colombo, W. DeWitt, M. Murayama, and M. A. Naughton; and in the graduate students involved directly in this work: John Hunt, Anthony Stretton, and, more recently, Bernard Moss and George Maniatis. So many ideas and experiments were contributed by them.

What I would like to do in this talk is to define some of the problems which must be faced by a biochemist with genetic leanings and an interest in developmental biology.

Through the work of many people, we know today a great deal about mutations in human and bacterial proteins. We have many examples, and we can describe these mutations in detail chemically. Even the list of abnormal human hemoglobins now numbers over 80—from so many different areas of the world that it reads like a travel catalogue! They illustrate different kinds of mutations: amino acid substitutions, deletions of one to five amino acids, crossing over as in hemoglobin Lepore, for instance. They illustrate the genetic events of gene duplication and the importance of these gene duplications in the evolution of a protein such as hemoglobin.

Two obvious lines of further work suggest themselves: One is concerned with the quantitative control of hemoglobin synthesis as opposed to the qualitative control demonstrated in the mutations. Second, changes are observed in hemoglobin types in many developing embryos; to study the control of hemoglobin synthesis is a useful technique for investigating the molecular basis of differentiation.

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## QUANTITATIVE CONTROL

The most obvious example of quantitative control of synthesis occurs with hemoglobin $A_2$, which amounts to only one-fortieth of the normal adult hemoglobin and whose control is accurate and strict in the normal human being. An unstable messenger RNA for hemoglobin $A_2$ has been suggested to account for the small amount of $A_2$ made, so unstable, perhaps, according to R. M. Winslow (unpublished observations), that hemoglobin $A_2$ is always made on a nascent messenger. Since it is supposed that this nascent messenger is easily destroyed, hemoglobin $A_2$ is kept down to a low level. Furthermore, when considering the rate of assembly of a hemoglobin molecule, we must consider the possibilities that there are control points in the rate of assembly of such a protein. This is an important general concept which has been investigated in the hemoglobins by Winslow and myself (Winslow and Ingram, 1966).

It is suggested from our experiments that a ribosome making hemoglobin travels faster along the mRNA for the first two-thirds of the path than along the last stretch. It is not enough to say that the messenger RNA is being read by a ribosome. We now have to be more sophisticated and ask whether the rate is uniform. How fast does it travel? What are the factors controlling this? Because here one might have an important mechanism controlling whether a protein is made at all or made in sufficient amount. We know that transfer RNA's are required and that some transfer RNA's are rare. It may be that at the point of control a rare transfer RNA is needed, and, since it is rare, the rate of production of the protein is slowed down (Itano, 1966). Yet another model suggests that heme has to be added to the growing peptide in order for synthesis to take place. The experiments of Levere and Granick (1965) show that heme synthesis is limiting in the synthesis of hemoglobin and that hemoglobin synthesis does not begin until heme is present. The production of heme itself is controlled by one of the enzymes—δ-aminolevulinic acid synthetase—involved in the biosynthetic pathway. Wilt (1962) has also shown that the protein portion is already present before heme synthesis begins in the developing chick embryo. So the control of hemoglobin synthesis might be the control of heme synthesis itself. Even if this is not an "all or none" control, it might well control the rate at which hemoglobin is made. This is the kind of experimental situation which people are beginning to examine very carefully now.

We usually assume, in any case, that when a message is present it will be read at maximum speed, but this is not necessarily so. Conditions in the cell might change in such a way as to influence the speed of translation of the message. That is about as far as one can go in defining the quantitative control of hemoglobin synthesis; a great deal of work will be required before we can give a precise description of the situation, as one would like to do, instead of dealing with the problem in a purely qualitative way.

## THE CONTROL OF EMBRYONIC HEMOGLOBINS

Now I would like to turn to the use of hemoglobin in the study of development. It is a convenient protein to work with, because a great deal is known about it and it is so easily isolated. It is of importance for the understanding of fetal and embryonic

development to be able to describe and explain the changes that take place in hemoglobin synthesis during embryonic development as well as all the other changes.

Let us ask a few questions about the developmental changes in hemoglobin synthesis. How many different hemoglobins are there in the development of an animal and what are their structural relationships? Second, how many distinct red cell types are there during development and where are they produced? This is the descriptive stage, quite difficult enough in itself, but next one reaches the even more difficult questions of why there are different hemoglobins and different cell types in the developing animal and how they arise. It will be a long time before we can begin to answer these questions. A few experiments which we have done are relevant.

*Tadpoles: Thyroxine Induces a Change to Frog Hemoglobin*

The American bullfrog tadpole, *Rana catesbeiana*, is a large animal with the great advantage that its metamorphosis is arrested when it is a few inches long and then does not start again naturally until a year or two later. During that time, thyroxine stimulates the development artificially so that one can trigger development at will. During the first 10 days of treatment with a rather high concentration of thyroxine, $5 \times 10^{-8}$ M, the ability of the circulating red cells to synthesize hemoglobin falls drastically; after about 8 or 9 days, depending upon the concentrations of thyroxine, it rises again (Moss and Ingram, 1965). Since red blood cells differentiate and mature in erythropoietic organs such as the liver and only enter the circulation when they are practically mature, the circulating cells are mostly mature cells no longer active in hemoglobin synthesis, with a very small proportion of recently released cells. In our experiments, radioactive amino acids are incorporated into hemoglobin by this small group of still immature cells; we are examining therefore not only the nature of the hemoglobin being made but also the release or nonrelease into the circulation of red cells in the last stage of maturation. Thyroxine has the double effect of preventing the release of the larval cell type containing tadpole hemoglobin and also of stimulating the maturation and release of cells synthesizing and containing frog hemoglobin. Originally, the tadpole's cells make tadpole hemoglobins, which are chemically distinct from frog hemoglobins. When the ability of the circulating blood to make tadpole hemoglobin falls, this means that, in the circulating blood, the immature tadpole cells have matured and lost their synthetic ability. After a week or so of treatment, a new population of cells is released which is able to make hemoglobin again, but now it is frog hemoglobin. The appearance of a new hemoglobin type in the blood has been triggered artificially by thyroxine.

Our first concern was to show that the radioactive hemoglobin synthesized by the new cells after thyroxine treatment is indeed frog hemoglobin (Moss and Ingram, 1968). This was found to be the case, using such techniques as polyacrylamide gel electrophoresis of the hemoglobins or of the peptide chains, Sephadex gel filtration (which is quite characteristic in this case, since the major frog hemoglobin dimerizes), and fingerprinting of trypsin digests.

Quite clearly, the major frog and tadpole hemoglobins have quite different peptide chains, separable by electrophoresis, and therefore we can formulate the thyroxine-induced change as involving the turning off of one set of structural genes and the

activation of a different set of structural genes. In this view, the larval and adult hemoglobin genes have their own switching control, distinct from the control of other cell constituents.

Another and possibly more realistic way of formulating a hypothesis is to suppose that the tadpole red cell epigenotype has a characteristic set of active genes and that the active tadpole hemoglobin genes are *merely a part of this set.* Similarly, the frog hemoglobin genes are merely a part of the frog red cell epigenotype. According to this second formulation, switching controls the epigenotype as a whole, and the effect on production of hemoglobin is a consequence.

In some systems, particularly the chick embryo, where the early and late cell types are easy to identify, the appearance of a new cell type is strongly correlated with appearance of a new hemoglobin type. Even so, it is going to be hard to design an experiment to distinguish between the two switching control systems mentioned above, unless perhaps we can stimulate hormonally or by transplantation the appearance of the unexpected combination of cell type and hemoglobin type.

The problem is complicated by the possibility that the switching of hemoglobin type may be a quantitative rather than a qualitative, or on-off, change. In the tadpole-frog metamorphosis, we always find in tadpole cells a very small amount of hemoglobin which might well be frog hemoglobin; the reverse is true of frog cells. The chemical nature of such small amounts is difficult to establish with certainty. Quite possibly, every tadpole cell makes a small percentage of frog hemoglobin; alternatively, the small amount of frog protein could be contained in a few "frog" cells only, leaving the intracellular switch as a complete qualitative change, but making the switch in cell type merely a quantitative change. Experiments are under way to settle this question in tadpoles.

In human development, where fetal and adult hemoglobins are easily distinguished even within a single cell, it is known that cells containing both types of hemoglobin are common during the transition period. These hemoglobins, of course, have one peptide chain—the $\alpha$ chain—in common, and they differ in this respect from the tadpole-frog hemoglobins. Nevertheless, it seems that, in the human case, partial switching is common at one stage, but it is not clear whether this is a partial switching of the whole epigenotype or only of the relevant $\beta$ and $\gamma$ peptide chain genes.

*Tadpoles: Thyroxine-induced Changes in Red Cell Type*

The changes in erythropoietic activity in the tadpole liver have been studied in relation to natural and thyroxine-induced metamorphosis (G. Maniatis and V. M. Ingram, unpublished observations). It seems that, contrary to the reports in the literature (Jordan and Speidel, 1923), the liver, rather than the kidney, is the major site of erythropoiesis. Prints of liver slices show the maturing and the circulating red cells very well. In natural metamorphosis, the proportion of immature cells (which already contain some hemoglobin, however) falls almost to zero; this is later followed by a rise back to the original level of 5–6%.

Although this finding fits well with the over-all picture of red cell changes in metamorphosis, it is not possible at the moment to classify these cells morphologically into tadpole or frog types. In natural metamorphosis, the immature cells are large and

oval, as could be expected from the shape of the mature tadpole or frog cell. However, if thyroxine is used to induce metamorphosis both in the liver and in the circulating blood, most of the new cells—frog-hemoglobin-containing cells—are round and rather small and contain relatively less hemoglobin (Moss and Ingram, 1968; De Witt, 1968). Although these small round cells contain and synthesize frog hemoglobin, they are a type not usually seen. It is not clear whether they are an intermediate in the normal maturation of frog red cells, forced to mature and be released by the thyroxine treatment, or whether they are an altogether different cell line, not normally differentiating into red cells but which are caused to do so by thyroxine. So far, experiments to reproduce this phenomenon in organ culture have failed.

*Chick Embryos: Developmental Changes in Hemoglobin and in Red Cell Types*

It is known from the work of several laboratories (summarized by Wilt, 1967, in an excellent review) that in the developing chick embryo four or five different hemoglobins appear in sequence. Nobody knows why the embryo should need different hemoglobin proteins at different stages of development; indeed, it is not known in the human case why we have first an embryonic hemoglobin, then a fetal, and finally an adult hemoglobin. There is no obvious reason why a different structural protein for the same function should be involved in the different developmental stages. Perhaps the important sequence is a sequence of cell types which happen to produce different hemoglobins as an accident of evolution.

In our own studies (H. Hagopian and V. M. Ingram, unpublished observations), we have concentrated on the developing chick embryo from 36 hr of incubation to 9 days. During that time, the early, or *primitive* (P), hemoglobin is the major component up to day 6. The picture then changes rapidly, and a more negatively charged *definitive* (D) hemoglobin predominates. According to Wilt (1967), these hemoglobins have only one of their peptide chains in common.

Other hemoglobins are seen before and after this period, but one can express the change for the period of 2–9 days' incubation by measuring the ratio of D/P hemoglobins after electrophoretic separation on, for example, polyacrylamide gels. The abrupt change is seen at the same time, on day 6, both in intact embryos and in organ cultures of the area opaca from 36-hr embryos. The ratio changes from 0.3 to 2 or even to 3.

At the same time, there is the well-marked change in red cell morphology, from the large round primitive erythrocyte to the much smaller oval definitive erythrocyte. Both cell types remain nucleated with small condensed inactive nuclei. It is interesting to speculate that the primitive cell type contains primarily the primitive hemoglobin and the definitive cells the D hemoglobin.

In cultures of these early blastoderms, we could show not only that the expected change in the ratio of D/P hemoglobins occurred at the same time as in vivo but also that puromycin or cycloheximide given after day 4 prevented the appearance of the definitive hemoglobin. It is not yet clear what happened to the cell morphology in these experiments.

What can one do to manipulate the system so as to see how the second hemoglobin and the second cell type appear? There are reports in the literature (Stockdale and

Topper, 1966) that in mammary gland epithelium in culture, some of the cells can be induced to differentiate further and produce the characteristic protein casein when challenged by the hormones insulin, hydrocortisone, and prolactin. However, it seems that DNA synthesis and/or cell division has to precede this hormone-induced differentiation.

If the same holds true for the chick embryonic hemoglobin change, then one would expect inhibition of DNA synthesis to prevent the appearance of the definitive hemoglobin. The agent used by us was a high concentration ($2 \times 10^{-3}$ M) of thymidine (Puck, 1964), which blocks DNA synthesis reversibly and which can be easily washed out again; normal synthesis then follows. Exposure of the 36-hr blastoderm to thymidine was for 24 hr, and this was followed by washing out the thymidine and by continued culture in control medium. The ratio of D/P hemoglobin changed just as it did in control cultures, but the change of the ratio was delayed by about 24 hr. The simplest explanation of these observations is that the cells which are going to make the later D hemoglobin have to divide, or at least synthesize DNA, before they are able to make the new hemoglobin, or maybe any hemoglobin. Perhaps it is only freshly synthesized DNA which can respond to whatever the inducing factors are. Clearly, the system requires a great deal of clarification.

## GENERAL PROBLEMS IN RED CELL DIFFERENTIATION

In formulating questions on the development of hemoglobin synthesis in embryos, I think one can make a few useful statements. It may be that the basic event of importance to the embryo is not a change in hemoglobin pattern but a sequence of distinctly different red cell populations and that the change in hemoglobin pattern is only a consequence of having successive and different red cell populations. These would differ in morphology, their site of production, their oxygen-carrying properties, and, as a corollary, in hemoglobin type. These cells are different epigenotypes, using the word as Abercrombie (1967) did; epigenotype may be defined here as a self-reproducing, regulatory mechanism that characterizes each of the different tissue types of an organism. It is thus a small part of a genotype. Therefore, when one talks about red cell populations, in the human case, for example, we would have first an embryonic epigenotype, a cell which appears to remain nucleated and which produces the characteristic $\epsilon$ peptide chain for the embryonic human hemoglobin. Next follows the fetal epigenotype which determines the kind of cell found in fetuses and which contains fetal hemoglobin; and finally the adult cell type carrying hemoglobins A and $A_2$.

Next there have to be regulatory mechanisms which control not only the characteristics of the cell type but also the degree to which a cell line, during development, has the ability to differentiate. For example, controls are needed for the timing of the change from the early primitive chick embryo cells to the later definitive type. It is a truism to state that early enough in a chick blastodisc all the cells have the potential to form any one of the cell types seen in the final organism. As they develop, different sets of cells with more restricted potential appear, and gradually, through a series of steps, the cells lose their ability to differentiate into distinct epigenotypes. It is entirely likely and widely believed that all these cells have DNA with the same sequence, that they all have the same genome. Presumably at the very beginning, those parts

of the cell genome which are concerned with keeping the cell alive and which insure its reproduction are active, but those other parts of the genome which have more specific functions are repressed. As the cell differentiates, different parts of the genome become active. As a line of cells differentiates toward a red cell type, those parts of the genome that make it a red cell will become active while at the same time the other parts which would have been active in, say, a liver cell must remain inactive. Two things have to occur—the activation of those parts of the genome which determine the general characteristic of the cell type and the inactivation, the continued inactivation, of the unwanted or uninduced sections of the genome. In advanced stages of differentiation, one has to lock in the epigenotype. How can this happen? One can think of a number of possible models: for instance, some external agent, say, a maternal hormone in the case of the human fetus, might be involved. The hormone would control the epigenotype but would have to be present continuously or at least as long as the cell continues to divide. Alternatively, an external agent might enter the cell at a crucial time or when the cells are "receptive," combine with the DNA, and cause a mistake at the next DNA replication, so that the part of the genome which is no longer wanted in a certain cell type cannot be transcribed. The miscoding would be passed on. This would be a permanent locking-in of an epigenotype which could only be reversed by a second miscoding.

In this connection, and in contradiction, the experiments of Harris at Oxford are extremely interesting. He has fused mature chick erythrocytes with HeLa cells so that the inactive pycnotic nucleus of the fully differentiated erythrocyte is now inside a HeLa cell. The erythrocyte nucleus swells and begins to make DNA and RNA. It is not yet clear whether hemoglobin is being made again, but it does produce chick-specific surface antigens. In another recent report, such heterokaryons of chick erythrocyte with human cells have been induced to produce chick-specific interferon, an activation of a previously inactive chick gene.

These experiments seem to answer "Yes" to the question, Can one reverse differentiation in a fully differentiated cell, such as in the red cell series? Furthermore, it is well known that in early stages of differentiation, reversal of differentiation is possible in many cases. In agreement with this idea of reversal of differentiation are Steward's fascinating experiments of growing whole carrot plants from single, differentiated phloem cells.

The statement has been made that cells which are fully differentiated and are making their characteristic product no longer divide, but this is clearly not the case, since we and other people have examples of red cells from (frog) bone marrow containing hemoglobin, yet clearly dividing.

In discussing activation and deactivation of structural genes and employing one's model-building ingenuity, one can arrive at many different solutions, of which we can discuss just one as it applies to a differentiating red cell series.

We might extend Baglioni's scheme for the production of human fetal hemoglobin (1963) in terms of the Jacob and Monod repressors and operators (Ingram, 1963). We would then postulate that in the "young" stem cell of the fetus there is present a genetically controlled repressor substance capable of repressing the operator locus which controls both the $\beta$ and the neighboring $\delta$ genes. These are therefore set to

"silent" while $\alpha$ and $\gamma$ genes become derepressed when this particular cell differentiates. Thus, $\alpha_2\gamma_2$ (fetal hemoglobin) is produced. However, this specific $\beta/\delta$ repressor is either unstable or becomes diluted out as the stem cell continues to divide. Eventually it is at a low level, and, when *this* cell differentiates, the repressor of $\beta$ and $\delta$ is inactive or absent and the $\beta$ and $\delta$ loci are derepressed. In addition, we must assume further that either a new repressor for the $\gamma$ operator begins now to be made or that the $\beta$-chain messenger RNA molecules which are produced themselves act as repressors on the $\gamma$ operator. This would be a kind of feedback situation.

In an alternative scheme, we might postulate that a repressor substance for the $\gamma$ operator is present all the time in these cells but that there is in addition another substance, possibly of low molecular weight, possibly some hormone, which combines with the repressor and modifies it so that the complex acts as a repressor for the $\beta/\delta$ operator but does not act on the $\gamma$ operator. During the maturation of the fetus, the stem cells gradually lose this second substance, this repressor modifier, and the repressor itself now acts to repress the $\gamma$-chain operator but is no longer active on the $\beta$ operator. Such a modified scheme would relieve the $\beta$-chain messenger RNA of its dual role and might also indicate a possible involvement of hormonal substances in the switch mechanism.

There is some slight evidence for the influence of hormones in the production of fetal hemoglobin, since there are instances in the literature of women with false or molar pregnancies who form small amounts of fetal hemoglobin in their blood during their "pregnancies." Removal of the mole eliminates fetal hemoglobin production. It is known that during such molar pregnancies there is considerable production of a variety of hormones, particularly gonadotropin. This is not to say, of course, that gonadotropin itself might be involved or indeed that any hormones are necessarily involved, but it does provide another view of this important problem.

As yet another alternative, we can picture that the repressor-modifier substance slowly increases during the change from "young" stem cells to "old" stem cells. Again, the modifier could well be a low-molecular-weight substance arriving in the cell from the outside, although this is a more difficult concept than the gradual loss of such material from the cell. Apart from this difficulty, the scheme works equally well with the unmodified repressor having specificity to combine with, and turn off, the $\beta/\delta$ operator and with the modified repressor having specificity only for the $\gamma$ operator.

The advent of an external agent does not have to be just a hormone produced from the outside, like thyroxine or maternal hormone. When cells are dividing and developing together, they may secrete substances which act on each other, and the activity of such substances might well depend on the concentration. This is a kind of field effect which has been postulated for the development of fetal tissues. In a cluster of cells, the ones at the center will have a different environment from the cells at the periphery, and by postulating the secretion of a material one can also postulate a differential effect on the cell population, depending on whether they are near the periphery or the center of such a cell population. In this model, the nature of the repressors changes during differentiation and the fetal genes become repressed and the

adult ones derepressed, thus producing a different epigenotype. This could occur in the same cell line or in different branches of the cell line.

Opposed to this view is the basis of the thymidine experiment I talked about earlier, an "internal clock," in which the differentiating cells "count" the number of cells divisions, perhaps by diluting a cell constituent. In a developing cell population, at the stage of the completely undifferentiated cell, most of the genome is repressed and the characteristic properties of the final cell line are not produced. The repressors active in this will be present early, but as the cell divides they would be diluted out, and the region of activity of the genome would change. Therefore, if one delays DNA synthesis, that is, cell division, one might delay this diluting effect and, therefore, delay the production of the change to a new hemoglobin type. That is just one of many possibilities.

SUMMARY

It is obvious that, in addition to working with whole animals, it will be essential to extend the investigation of differentiating systems to in vitro work with cell or organ culture. Only in this way can we find out the relationship between the molecular metabolic events within a cell and the process of differentiation, particularly the role of external inducers or inactivators.

The red cell series, though by no means the only system, is particularly well suited to such studies because of their convenient morphological features and the characteristic hemoglobin proteins. It will be possible, one hopes, to investigate the reactivation of nuclei and thereby their original inactivation, to ask whether the same cell line can differentiate first to one cell type and then to another or whether these are separate branches, to determine the chemical nature and mode of action of the agents involved, and to look at the role of the environment provided by a particular organ or site in deciding the course of differentiation. This last question might best be asked in a situation where the "choice" is between two similar cell types.

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